

Separation of Proteins and Peptides on Discovery® BIO Wide Pore (300Å) C18, C8 and C5 Phases

Abstract

When performing protein and peptide separations, scientists prefer HPLC bonded phases to be stable and reproducible while exhibiting high resolution. In this article, we introduce three new reversed phases, C18, C8 and C5, which have been specifically developed for those needs. The C18 and C8 phases demonstrate high resolution peptide mapping and purification. The C5 phase exhibits enhanced stability and is excellent for protein separation. All of the phases show high lot-to-lot reproducibility.

Peptide Mapping and Purification with C18 and C8 phases

Peptide and protein chemists use C18 or C8 phases for peptide mapping and purification. However, problems of peak tailing or low resolution may be encountered due to secondary chromatographic effects. Some unnecessary active sites, such as active silanol groups and metal impurities, may still remain on the surface. This is especially critical for peptide mapping because it often requires resolving more than a hundred peaks in one hour. Figure A presents a comparison of a peptide map with well resolved peaks on Discovery BIO Wide Pore C18 phase vs. the same application on a prominent competitor's column.

Protein Analysis and Purification with C5 Phase

In protein analysis and purification, possible sample denaturation is a consideration. For this reason, we would choose to use a short alkyl chain phase, such as C4, for the application. This may reduce the length of time the sample is retained on the column and lessen the amount of the stronger organic solvent needed. However, C4 is very susceptible to acid hydrolysis and may not remain stable over time. To alleviate this problem, Supelco has developed a new short alkyl chain C5 phase. It demonstrates almost identical selectivity to C4, but is substantially more stable, translating into longer column life. Figure B compares the stabilities between Discovery BIO Wide Pore C5 and a conventional C4 phase.

Reproducibility of the Phase

Reproducibility is another key factor to consider when selecting a column, and is generally discussed in terms of run-to-run, column-to-column, and lot-to-lot (or batch-to-batch).

Variation in run-to-run reproducibility is generally very low, provided the HPLC instrumentation is well maintained and the bonded phase is stable. Differences in column-to-column re-

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Figure A. Tryptic Digest of Carboxymethylated Apohemoglobin on Discovery BIO Wide Pore C18 vs. Competitor

Column: 15cm x 4.6mm; 5µm
Mobile Phase: (A) 95:5, (0.1% TFA in H₂O):
(0.1% TFA in MeCN)
(B) 50:50, (0.1% TFA in H₂O):
(0.1% TFA in MeCN)
Gradient: 0-100% B in 65 min
Flow Rate: 1mL/min
Temp: 30°C
Detection: 215nm
Injection: 50µL

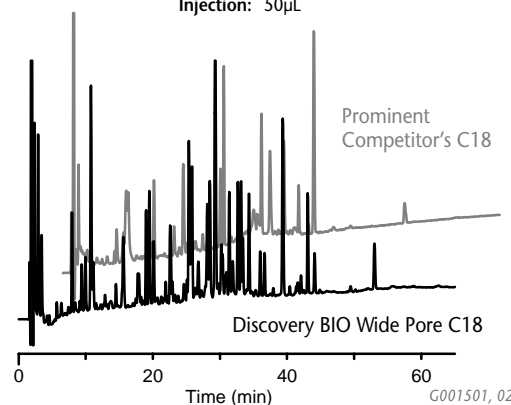
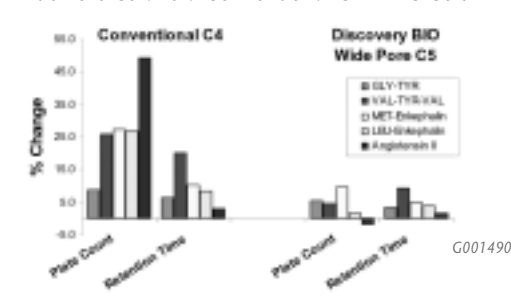


Figure B. Stability Comparison of Discovery BIO Wide Pore C5 and a Conventional C4 HPLC Column



producibility within the same lot are also negligible with today's standardized column packing and manufacturing processes. Rather, most reproducibility problems arise from lot-to-lot variances. Factors influencing lot-to-lot reproducibility include differences in silica lots, reagent lots, other processing materials, and operators. However, when strictly controlled, those effects can be greatly minimized. Figure C (page 4) presents the results of a peptide test mix performed on three lots of Discovery BIO Wide Pore C5. The RSD of the retention time of last peak is less than 2%.

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NEW PRODUCTS

NEW Discovery BIO and HS Columns

Supelco introduces six new phase chemistries into the Discovery family of HPLC columns. In order to better service the diverse needs of today's researcher, each phase chemistry is available in three different particle sizes. We are now offering Discovery BIO for biomolecule analysis and purification. These wide pore (300Å) columns are available in three different phase chemistries: C18, C8, and C5. In addition to BIO, the HS (120Å) line of columns has grown to include three new unique phases in addition to time tested C18. The entire HS line is now available in the following bonded phase chemistries: C18, PEG, and F5.

Discovery BIO WidePore C18 (3µm, 5µm & 10µm)

- Excellent resolution for synthetic peptide analysis and peptide mapping
- Highly stable in alkaline pH mobile phase and constant column selectivity over time
- Scalable from analytical to prep
- Excellent lot to lot column reproducibility for robust assays of synthetic peptides and peptide mapping
- Ideal for LC/MS applications - No detectable bleed

☎ For more information, request T401097.

Discovery BIO WidePore C8 (3µm, 5µm & 10µm)

- Intermediate hydrophobicity (less than C18; greater than C4/C5 phases) offers an excellent starting point for method development
- Ideal for the analysis and purification of peptides, polypeptides and smaller proteins
- Scalable from analytical to prep
- Excellent column stability at alkaline pH and constant column selectivity over time
- Well suited for LC/MS applications

☎ For more information, request T401098.

Discovery BIO WidePore C5 (3µm, 5µm & 10µm)

- Excellent performance for large polypeptide and protein analysis and purification
- Faster separations for hydrophobic peptides compared to C18
- Increased column stability in acidic and alkaline mobile phase compared to conventional C4
- Scalable from analytical to prep
- Excellent lot to lot column reproducibility for robust assays of biotechnology products

☎ For more information, request T401099.

Discovery HS C18 (3µm, 5µm & 10µm)

- Specifically developed for pharmaceutical analysis and purification
- Ideal for LC/MS applications - No detectable bleed as independently tested
- Highly stable to ensure excellent run-to-run and lot-to-lot reproducibility and long column life
- Scalable from analytical to prep

☎ For more information, request T401095.

Discovery HS PEG (3µm, 5µm & 10µm)

- A unique polyethylene glycol reversed phase specifically developed for pharmaceutical analysis and purification
- Unique selectivity and faster separations of polar compounds compared to C18 phases
- Outstanding performance for phenolic compounds offers unique retention and selectivity and faster analysis compared to C18
- Scalable from analytical to prep
- Highly stable to ensure excellent run-to-run and lot-to-lot reproducibility and long column life

☎ For more information, request T401100.

Discovery HS F5 (3µm, 5µm & 10µm)

- A unique pentafluorophenylpropyl terminated reversed phase column specifically developed for pharmaceutical analysis and purification
- Unique retention and selectivity compared to C18 phases
- Scalable from analytical to prep

☎ For more information, request T401096.



All literature mentioned in this issue can be obtained from the website, sigmaaldrich.com/TheReporter, by completing the Literature Request section on the reply card, or by calling our Technical Service Department.

Analysis of Drugs Coming Off Patent Using Discovery HS C18 Columns

Over the next three years, fifteen well-known pharmaceutical compounds with substantial worldwide sales will be coming off patent. Ulcers, depression, hypertension and bacterial infections are a number of the illnesses treated by these compounds. Although many of these illnesses are not medically related to each other, the compounds used to treat these ailments do have one commonality between them. These pharmaceutical compounds may all be analyzed by reversed-phase liquid chromatography (RPLC). The use of RPLC will help the manufacturer determine compound purity and will also help the clinical chemist determine that the correct dosage to the patient is being administered.

Supelco has developed methods for the analysis of a number of drugs coming off patent. Many of these methods utilize the Discovery HS (high surface area) C18 column. Methods may be run on either the 3 μ m or 5 μ m HS C18; both size particles are amenable to LC/MS (Mass Spectrometry). In this application report we highlight two new applications on the 5 μ m HS C18 column using MS detection.

Figure E shows the separation of Prilosec® (Omeprazole) using a mass spectrometer friendly mobile phase. In addition, Clarithromycin has been added to the sample as this compound may be given along with the Prilosec. Below

the chromatogram (shaded peak is Prilosec), the mass spectrum for this compound is shown. Note that the separation is accomplished in less than 6 minutes with excellent chromatographic resolution.

Figure F shows the separation of Prozac® (Fluoxetine HCl) along with Diazepam which is sometimes administered along with the Prozac. In this case the separation takes only four minutes to complete. The mass spectrum is located below the chromatogram of the shaded Prozac peak.

In each of these applications a 2.1mm ID column was used with no split of the column effluent into the mass spectrometer. It is critical that the HPLC system is plumbed correctly to minimize dead volume.

In this application report, a Quality Assurance (QA) LC method has been demonstrated for each compound. In addition, an LC method showing both the pharmaceutical coming off patent and a compound most commonly co-administered with the major drug of interest would be of value to the clinical chemist. Finally, a mass spectrometer coupled to an LC provides the superior method of detection using the proven no bleed chemistry of the Discovery HS C18.

☞ For more information, request T401095 and T201005.

Figure E. Total Ion Chromatograms of Prilosec

Column: Discovery HS C18, 15cm x 2.1mm, 5 μ m;
Mobile Phase: 60% ammonium acetate, adjusted to pH 5 with 28% acetic acid: 40% MeCN; **Flow Rate:** 0.2mL/min; **Temp:** 30°C; **Detection:** MS in ESI (+) ion mode; **Injection:** 2 μ L

1. Prilosec (Omeprazole) (100 μ g/mL)
2. Clarithromycin (100 μ g/mL)

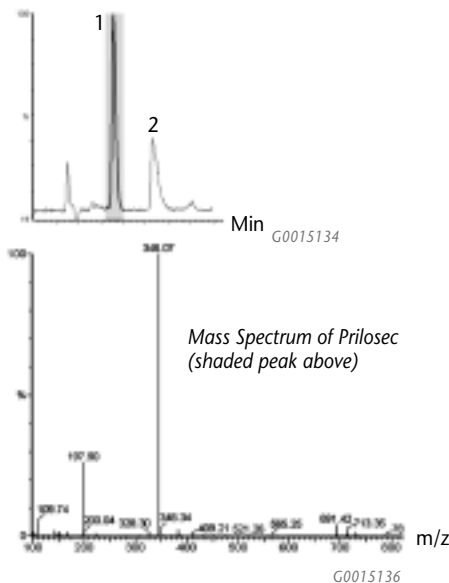
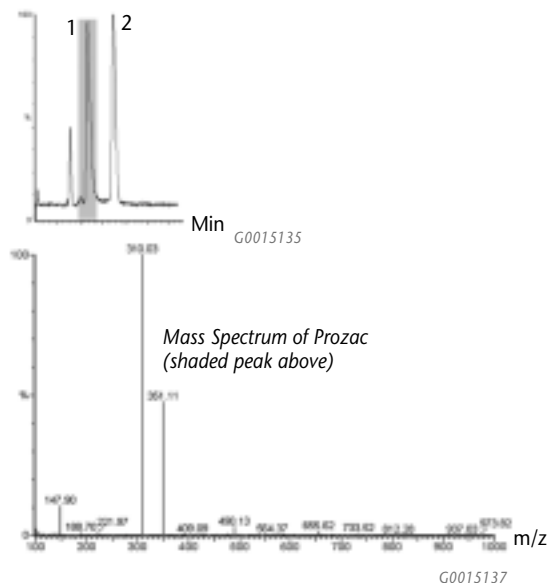


Figure F. Total Ion Chromatograms of Prozac

Column: Discovery HS C18, 5cm x 2.1mm, 5 μ m;
Mobile Phase: 30% ammonium acetate, adjusted to pH 5 with 28% acetic acid: 70% MeCN; **Flow Rate:** 0.2mL/min; **Temp:** 30°C; **Detection:** MS in ESI (+) ion mode; **Injection:** 2 μ L

1. Prozac (Flouxetine Hydrochloride) (50 μ g/mL)
2. Diazepam (50 μ g/mL)



Trademarks and Registered Trademarks:

Discovery- Sigma-Aldrich Co.
Prilosec- Astra AB.
Prozac- Dista Products Company,
Division of Eli Lilly and Company

Separation of Proteins and Peptides...

(continued from page 1)

Conclusion

To separate proteins and peptides by reversed phase chromatography, consideration should be given to column resolution, stability and reproducibility. Discovery BIO Wide Pore C18

and C8 have been developed specifically to address these issues. In protein separations, C5 is generally a better choice than C4 because it maintains the advantages and selectivity of a short alkyl phase while exhibiting superior stability.

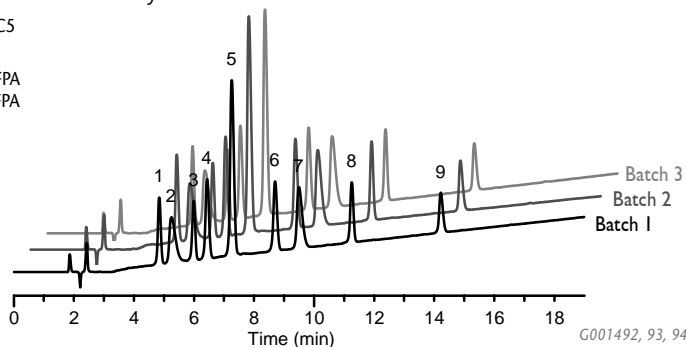
For more information, request T401095, T401096, T401097, T401098, T401099, T401100.

Figure C. Results of a Peptide Mix on Three Lots of Discovery BIO Wide Pore C5

Lot to Lot Reproducibility of Discovery BIO Wide Pore C5

Column: 15cm x 4.6mm columns, 5 μ m
Mobile Phase: A: 81:19:0.1, water:acetonitrile:PFPA
B: 62:38:0.1, water:acetonitrile:PFPA
Gradient: 0 to 100%B in 19 min, 1mL/min
Temp: 30°C, UV 215nm, 10mL injection

1. Arg⁸-vassopressin
2. Brandykinin, fragment 1-5
3. Oxytocin
4. Met-enkephalin
5. Luteinizing Hormone Releasing Hormone
6. Leu-enkephalin
7. Brandykinin
8. Bombesin
9. Substance P



CASE STUDY 3

Scale-up of Gradient Method

Scale-up of a gradient method accounts not only for changes in capacity and flow rate (as in isocratic separations) but also for gradient volume. Particle size and column length are kept constant. However, in this example, particle size is varied for demonstrating matched selectivity, Discovery BIO Wide Pore C18. Scale-up of gradient methods will require determination of system dwell volume and column volume. Column volume does not refer to the calculated geometric volume of the empty hardware, but refers to the "extra-particle" volume (or volume of mobile phase) within the column. This is best determined by injection of an unretained compound and noting its retention time.

One key concept in gradient method development is that of gradient volume: the total volume of mobile phase pumped through the column from the start of the gradient to the end of the gradient. This is in terms of column volumes, not the absolute volume. Thus, the gradient volume is referred to in units of column volumes.

Calculations for scale-up are as follows (for columns of equivalent length).

$$\text{Flow rate: } f_2 = f_1 \times (r_2/r_1)^2$$

$$\text{Gradient volume: } v_2 = v_1 \times (v_{02}/v_{01})$$

$$\text{Gradient delay: } d_2 = d_1 \times (v_{02}/v_{01})$$

Note: gradient delay always includes d_w ; for initial method development on analytical column, if no intentional gradient delay is part of the method, $d_1 = d_w$.

$$\text{Sample capacity (to an approximation):}$$

$$m_2 = m_1 \times (v_{02}/v_{01}).$$

Where f is geometric flow rate, r is column diameter, d_w is system dwell volume, v is gradient volume, d is gradient flow rate, v_0 is the column volume (elution volume of unretained compound), m is sample mass.

The successful application of this scale-up methodology is illustrated in Figure D, in which selectivity of the peptide sample is matched across the range of particle sizes from high-performance analytical to preparative, with Discovery BIO Wide Pore C18.

For more information, request T200010.

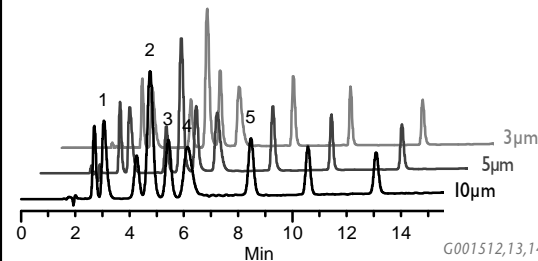
Figure D. Scale-up of Gradient Separation from Analytical to Prep

Mobile Phase: A: 80:20, (water/0.1% TFA) : (MeCN/0.1% TFA), B: 66:34, (water/0.1% TFA) : (MeCN/0.1% TFA), Flow Rate: 6.02cm/min, Temp: 30°C, Det: 215nm, Sample: Sigma Cat. No. P2693, 0.13g/L of each component

Column Parameters & Run Conditions:

Column	v_0 (mL)	Injection (μ L)	Flow (mL/min)
3 μ m, 15cm x 4.6mm	1.64	5.0	1.00
5 μ m, 15cm x 4.6mm	1.71	5.0	1.00
10 μ m, 15cm x 10mm	8.01	25	4.73

Gradient:	Column Volumes	%A	%B
	0	100	0
	2	100	0
	9	0	100



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